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Application 218093/1998

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[Title of the Invention]	Cancer antigens based on products of tumour suppressor gene *WT1*
[No. of Claims]	7
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[Document] Abstract 1

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Application 218093/1998

[Document] **Specification**

[Title of the Invention] **Cancer antigens based on products of cancer suppressor gene *WT1***

[Claims]

[Claim 1] A cancer antigen in which a product of cancer suppressor gene *WT1* or a partial peptide thereof is an active ingredient.

[Claim 2] A cancer antigen as described in Claim 1, in which the active ingredient is a peptide comprising 7-30 consecutive amino acids in SEQ ID NO: 1, including at least one anchor amino acid selected from Met, Asn and Ile, or a peptide comprising 7-30 consecutive amino acids in SEQ ID NO: 2, including at least one anchor amino acid selected from Met, Leu and Val.

[Claim 3] A cancer antigen as described in Claim 1 or 2, in which the aforementioned antigen is an antigen for cancer which causes high expression of cancer suppressor gene *WT1*.

[Claim 4] A cancer antigen as described in Claim 1 or 2 in which the aforementioned cancer is leukaemia, a myelodysplastic syndrome, malignant lymphoma, multiple myeloma, stomach cancer, bowel cancer, lung cancer, breast cancer, germ cell cancer, liver cancer, skin cancer, bladder cancer, uterine cancer, cervical cancer or ovarian cancer.

[Claim 5] A cancer antigen as described in any of Claims 1-4 in which the aforementioned peptide is any of:

K^b 45 Gly Ala Ser Ala Tyr Gly Ser Leu (SEQ ID NO: 3)

K^b 330 Cys Asn Lys Arg Tyr Phe Lys Leu (SEQ ID NO: 4)

D^b 126 Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 5)

D^b 221 Tyr Ser Ser Asp Asn Leu Tyr Gln Met (SEQ ID NO: 6)

D^b 235 Cys Met The Trp Asn Gln Met Asn Leu (SEQ ID NO: 7)

WH 187 Ser Leu Gly Glu Gln Gln Tyr Ser Val (SEQ ID NO: 8).

[Claim 6] A cancer antigen as described in Claim 5 in which the aforementioned peptide is:

D^b 126 Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 5), or

WH 187 Ser Leu Gly Glu Gln Gln Tyr Ser Val (SEQ ID NO: 8).

[Claim 7] A cancer vaccine including a cancer antigen described in any of Claims 1-6.

[Detailed Description]

[0001]

[Technical Field of the Invention]

The present invention relates to cancer antigens based on products of the Wilms' tumour suppressor gene *WT1*. These cancer antigens are useful as cancer vaccines against leukaemia, myelodysplastic syndromes, multiple myeloma and malignant lymphoma and other cancers of the blood, or solid cancers such as stomach cancers, bowel cancers, lung cancers, mammary gland cancers, germ cell cancers, liver cancers, skin cancers, bladder cancers, prostate cancers, uterine cancers, cervical cancers and ovarian cancers for example, as well as all cancers which express *WT1*.

[0002]

[Background Art]

The immune mechanisms for eliminating foreign substances are commonly classified into humoral immunity, due to macrophages which recognize antigens and function as antigen presenting cells, helper T lymphocytes which recognize antigens presented by said macrophages and activate T other lymphocytes etc.,

by producing lymphokines, and B lymphocytes which differentiate into antibody-producing cells under the action of said lymphokines, and cellular immunity, in which killer T lymphocytes which have differentiated on being with presented with an antigen then attack and destroy the target cells.

[0003]

At the present moment in time, immunity to cancer is thought to be principally due to cellular immunity mediated by killer T cells. In cancer immunity due to killer T cells, precursor T cells recognize a cancer antigen presented in the form of a complex between the cancer antigen and the major histocompatibility complex (MHC) class I and differentiate and proliferate to produce killer T cells which attack and destroy the cancer cells. In this process, the MHC class 1 antigen-cancer antigen complex is presented on the cell surface, and this is the target for the killer T cells (*Curr. Opin, Immunol.*, **5**, 709 (1993); *Curr. Opin. Immunol.*, **5**, 719 (1993); *Cell* **82**, 13 (1995), *Immunol. Rev.* **146**, 167 (1995)).

[0004]

The aforementioned cancer antigens presented by MHC class I antigens on cancer cells are thought to be peptides comprising approximately 8-12 amino acids produced as a result of intracellular protease processing of antigen protein synthesized in the cancer cells (*Curr. Opin. Immunol.* **5**, 709 (1993); *Curr. Opin. Immunol.* **5**, 719 (1993); *Cell* **82**, 13 (1995); *Immunol. Rev.* **146**, 167 (1995).

Currently, various cancers have been screened for antigen protein, but few cancer-specific antigens have been demonstrated.

[0005]

The Wilms' tumour suppressor gene *WT1* (gene *WT1*) was isolated from chromosome 11 p13 and identified as a gene causing Wilms ' tumour as the result of analysis of WAGR syndromes complicated by Wilms' tumour, aniridia,

genitourinary abnormalities and retarded neurotransmission, etc., (Gessler, M., *et al. Nature* Vol. 343 p. 774-778 (1990); the genomic DNA comprises 10 exons of approximately 50 kb, and its cDNA is of approximately 3 kb. The amino acid sequence deduced from the cDNA is shown in SEQ ID NO: 1 (*Mol. Cell. Biol.*, **11**, 1707 (1991)).

[0006]

Gene *WT1* is highly expressed in human leukaemia and proliferation of leukaemia cells is suppressed when the cells are treated with WT1 antisense oligomers (Japanese Laid-Open Patent 104627/1997), indicating that gene *WT1* acts to promote the proliferation of leukaemia cells. Moreover, gene *WT1* is also highly expressed in stomach cancer, bowel cancer, lung cancer, breast cancer, germ cell cancer, liver cancer, skin cancer, bladder cancer prostate cancer, uterine cancer, cervical cancer and ovarian cancer and other solid cancers (Japanese Patent Application 191635/1997), confirming that gene *WT1* is a new tumour marker in leukaemia and solid cancers. However, it has not been demonstrated that products of *WT1* gene expression are useful cancer-specific antigens for anticancer vaccines.

[0007]

[Problem Which The Invention Is Intended To Solve]

Therefore, the object of the present invention is to confirm that products of gene *WT1* expression are possible cancer antigens, and to offer novel cancer antigens.

[0008]

As the result of studies designed to solve the problem above, the present inventors have synthesized peptides of 7-30 consecutive amino acids within the amino acid sequence of products of expression of gene *WT1*, including at least 1

amino acid predicted to function as an anchor amino acid in binding with murine and human MHC class I and MHC class II, and have confirmed that these peptides bind with MHC class I proteins; and have also confirmed that binding with an MHC class I antigen induces killer T cells, and leads to an advantageous cytotoxic effect on target cells, thereby perfecting the present invention.

[0009]

Therefore, the present invention offers cancer antigens which include a product of expression of the murine *WT1* gene or a portion thereof. In a preferred form, the present invention offers cancer antigens in which the active ingredient is a peptide comprising 6-30 amino acids within the amino acid sequence shown in SEQ ID NO: 1 corresponding to WT1 cDNA, including at least one amino acid selected from a set comprising Met, Asn and Ile, assumed to function as anchor amino acids for binding with MHC class I antigens.

Anchor peptide, ie Met, Asn 16 delete from PG

[0010]

Moreover, the present invention also offers cancer antigens in which the active ingredient in a peptide comprising 7-30 amino acids in the amino acid sequence shown in SEQ. ID NO: 2 corresponding to human WT1 cDNA, including at least one amino acid selected from a set comprising Met, Leu and Val, assumed to function as anchor amino acids for binding with MHC class I antigens.

The present invention also offers cancer vaccines which include a cancer antigen above.

[0011]

[The Preferred Mode for Carrying Out the Invention]

In the present invention, the selection of peptides expected to be selective for murine MHC class I K^b or D^b and human HLA A0201 and to have high affinity

for these was fundamental in designing the cancer antigen peptides.

From *Immunogenetics* Vol. 41, pp. 178-228 (1995), Phe and Tyr at 5 and Leu and Met at 6 were expected to be anchor amino acids for binding to K^b; and Asn-5 and Met and Ile at 9 were expected to be anchor amino acids for binding to D^b.

[0012]

In addition, the size of cancer antigen peptides presented by MHC class I is known to be about 8-12 amino acids, and therefore the cancer antigen peptides of the present invention are peptides comprising 7-30 consecutive amino acids in the amino acid sequence shown in SEQ ID NO:1 produced by gene *WT1*, including at least one of Met, Asn and Ile amino acid. The number of amino acids is preferably 8-12 – 8 to 9, for example.

[0013]

In the present invention, the peptides of 8 amino acids below were used as specific examples of peptides binding to MHC class I K^b:

K^b 45 Gly Ala Ser Ala Tyr Gly Ser Leu (SEQ ID NO: 3)

K^b 330 Cys Asn Lys Arg Tyr Phe Lys Leu (SEQ ID NO: 4)

and the peptides of 9 amino acids below were used as peptides binding to MHC class I D^b:

D^b 126 Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 5)

D^b 221 Tyr Ser Ser Asp Asn Leu Tyr Gln Met (SEQ ID NO: 6)

D^b 235 Cys Met The Trp Asn Gln Met Asn Leu (SEQ ID NO: 7).

The amino acids underlined in the sequences above are the amino acids predicted to function as anchor amino acids.

[0014]

The ability of these peptides K^b 45 and K^b 330 to bind with MHC class I K^b and the ability of peptides D^b126, D^b 221 and D^b 235 to bind with MHC class I D^b were determined using a cell line which expresses K^b and D^b (RMA-S) without antigen markers (empty).

Thus, MHC class I was highly expressed by culturing RMA-S at 26° C, and then the cultured cells were incubated for 1 hour at 37°C with solutions of the test peptides. As a result MHC molecules not bound to a peptide became unstable and were lost from the cell surface, leaving only peptide-bound MHC class 1 molecules. The RMA-S cells were then stained with fluorescently labelled monoclonal antibodies which recognized MHC class I (K^b or D^b). Finally, binding dissociation constants were calculated from the average quantity of fluorescence per cell, found by FACS assay (*Immunol Lett.* **47**, 1 (1995)).

[0015]

The results obtained were as follows.

K^b 45 -4.5784838 (log)

K^b 330 5.7617732

D^b 126 -6.2834968

D^b 221 -5.7545398

D^b 235 -6.1457624

From results above, that all of the peptides showed strong to moderate binding affinity (kd value) for K^b or D^b; however, the peptide which showed the highest affinity, D^b 126, was used in subsequent experiments.

[0016]

From *Immunogenetics* Vol. 41, pp.178-228 (1995), it was predicted that in man the anchor amino acids for binding to HLA-A0201 would be Leu and Met 2

from the N-terminus and Val and Leu 9 from the N terminus. Accordingly, two peptides comprising 9 amino acids from within the amino acid sequence for human WT1 protein (*Mol. Cell. Biol.* Vol., 11, pp. 1707-1712 (1991)) (SEQ ID NO: 2) were synthesized which met the conditions above.

[0017]

D^b 126 Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 5)

(The same as the sequence of D^b 126 in the mouse)

WH 187 Ser Leu Gly Glu Gln Gln Tyr Ser Val (SEQ ID NO: 8)

(The underlining indicates the anchor amino acids.)

The peptides above were tested for their ability to bind with HLA-A0201 by the following method.

The peptides above were incubated at 37° C for 1 hours with empty T2 cells having HLA-A*0201 (*J. Immunol.*, **150**, 1763 (1993); Blood **88**, 2450 (1996)), and then the T2 cells were stained with fluorescently labelled monoclonal antibodies which recognized HLA-A2.1, and the binding dissociation constant was calculated from the average fluorescence per cell determined by FACS.

[0018]

Binding capacity

Peptide	Kd (M)
D^b 126	1.89×10^{-5}
WH 187	7.61×10^{-6}

Both peptides showed at least moderate binding affinity.

The experiments below were performed using Db 126 and WH 187 above as peptides corresponding to human MHC.

[0019]

The present invention also relates to cancer vaccines in which the active ingredient is an aforementioned antigen. These vaccines can be used to prevent or treat cancers, including leukaemia, myelodysplastic syndromes, multiple myeloma and malignant lymphoma and other cancers of the blood, or stomach cancer, bowel cancer, lung cancer, breast cancer, germ cell cancer, liver cancer, skin cancer, bladder cancer, prostate cancer, uterine cancer, cervical cancer, ovarian cancer and other solid cancers, for example, by raising the level of expression of gene *WT1*. These vaccines can be administered orally or non-orally – for example by intraperitoneal administration, subcutaneous administration, intradermal administration, intramuscular administration, intravenous administration intranasal administration or by some other route.

[0020]

In addition to the peptide administered as the aforementioned active ingredient, the vaccine can also include a pharmaceutically permitted carrier, for example a suitable adjuvant, for example a mineral gel such aluminium hydroxide, a surfactant such as lysolecithin or a pluronic polyol, a polyanion, peptide or oil emulsion/suspension. Or it can be mixed into liposomes, include a polysaccharide and/or other aggregates included in vaccines. The dose will generally be 0.1 μg to 1 mg/kg per day.

[0021]

[Examples]

Next, the peptides of the present invention will be shown to be useful as cancer antigens and cancer vaccines by means of practical examples.

Example 1

C57BL/6 mice were immunized by twice weekly intraperitoneal injection of 100 μg of peptide D^b 126, 200 μg of porcine lactate dehydrogenase (LDH) and

0.5 ml of Freund's incomplete adjuvant. One week after immunization, the spleens were removed from the mice to prepare a splenocyte suspension. Meanwhile, irradiated splenocytes from mice of the same strain pulsed with peptide D^b 126 were incubated for 30 minutes at 37 °C with a solution containing the peptide at 50 μg/ml.

[0022]

The aforementioned immunized splenocytes and irradiated splenocytes were mixed and cultured together for 5 days to induce killer T cells. A killing assay was performed using the conventional method with europium-labelled EL-4 cells (expressing K^b and D^b) pulsed with peptide D^b 126 (incubated for 30 minutes at 37°C with a solution of peptide at 100 μg/ml) as target cells, by the following procedure (Table 1).

As a result, there was a cytotoxic effect with D^b 126-pulsed EL-4 target cells, but hardly any cytotoxic effect was seen in EL-4 cells that had not been pulsed with D^b 126.

[0023]

[Table 1]

Table 1

	Mouse A	Mouse B
Peptide +	76.6%	37.2%
Peptide -	4.9%	0.9%

E/T 40:1

[0024]

Next the expression of CD4 and CD8 in splenocytes which showed a significant cytoxic effect in the killing assay was analysed by flow cytometry after staining with fluorescently-labelled anti-CD4 anti bodies or anti-CD8 antibodies.

The results, in Figure 1, showed an increase in $CD8^+$ cells, typically killer T cells, in splenocytes immunized with peptide D^b 126 compared with non-immunized irradiated cells, with the increase reversing the proportion of $CD8^+$ cells relative to $CD4^+$ cells, typically helper T cells.

[0025]

Example 2

Dendritic cells (DC) were prepared as follows from the bone marrow of C57BL/6 mice. Myelocytes were cultured by the usual method in the presence of GM-CSF, to prepare bone marrow dendritic cells (*J. Exp. Med.* **182**, 255 (1995)).

Seven-day cultured dendritic cells were incubated for 3 hours with OVAII (Ovalbumin II) at 10 μM and peptide D^b 126 at 1 μM, and then washed.

[0026]

The DC cells above were then injected into the skin of the footpads and hands of C57BL/6 mice; on day 5, the associated lymph nodes were removed and a cell suspension was prepared. Meanwhile, B7.1-RMA-S cells pulsed with peptide D^b 126 and irradiated (RMA-S cells transfected with the gene coding the co-stimulatory molecule B7.1) were prepared.

[0027]

The above lymph node cell suspension and B7.1-RMA-S cells were then mixed and restimulated by culturing *in vitro*.

Then, on day 5 of *in vitro* restimulation a killing assay was performed, targeting ^{91}Cr-labelled RMA-S cells. Use of 1/8 of all the lymphocytes recovered on day 5 of restimulation as effector cells gave the maximum E/T ratio (1.0).

[0028]

As shown in Figure 2 and Figure 3, effector cells from lymph nodes of mice

immunized with peptide D^b 126 killed target cells pulsed with said peptide, whereas cells that were not pulsed with said peptide were not killed.

In addition, when the ratio of $CD4^+$ cells and $CD8^+$ cells was analysed by flow cytometry as in Example 1, CD4 : CD8 = 1 : 1.4-1.7 with an increase in $CD8^+$ cells among cells from mice immunized with peptide D^b 126 compared with non-immunized mice (control) (in control cells the ratio was approximately 2:1), with the ratio of $CD4^+$ cells and $CD8^+$ cells being reversed among immunized cells.

[0029]

Example 3

5×10^4 T2 cells irradiated after incubation for 1 hour with peptide D^b 126 or WH 187 (40 μg/ml) and 1×10^6 healthy human peripheral monocytes having HLA-A* 0201 were cultured together. After 1 week, T2 cells irradiated after incubation for1 hour with the peptide (20 μg/ml) were added to the above joint culture, and restimulated. From the following day human IL-2 was added to the culture solution (final solution 100 JRU/ml).

[0030]

Stimulation with T2 cells irradiated after pulsing with the peptide was repeated 5 more times, and then killing assays were performed with T2-cells pulsed with the peptide or unpulsed T2-cells as the target. Induction of CTL surface markers was also analysed by FACS.

The killing assay was performed by the usual method, with europium-labelled T2-cells pulsed with peptide used as the target.

Effector : target ratio (E/T ratio) was 10:1

Joint culture time: 3 hours.

Peptide concentration in the culture solution : 5 μ g/ml

[0031]

The results are shown in Figure 4. Figure 4 A shows the cytotoxic effect of CTL induced using peptide D^b 126 on T2-cells pulsed with peptide D^b 126; and Figure 4 B shows the cytotoxic effect of CTL induced using peptide WH 187 on T2-cells pulsed with peptide WT 187.

In both cases a more intense cytotoxic effect was seen in T2-cells pulsed with peptide.

[0032]

The results of FACS analysis are shown in Figure 5 to Figure 10. Figures 5-7 show the results for CTL induced by peptide D^b 126; nearly all cells were $CD8^+$. Figures 8-10 show the results for CTL induced by peptide WH 187. There were approximately equal numbers of $CD4^+$ and $CD8^+$ cells.

The results above demonstrate definitively that peptides of the present invention functioned as cancer antigens, causing the induction and proliferation of killer T cells against cancer cells (T cells harmful to cancer cells). Therefore, the cancer antigen peptides of the present invention are useful as cancer vaccines against leukaemia and solid cancers by raising expression of gene *WT1.*

[0033]

[Sequence listing]

.....

[0034]

.....

[0035]

.....

[0036]

.....

[0037]

.....

[0038]

.....

[Simplified Explanation of the Drawings]

[Figure 1]

Figure 1 is a graph showing the proportions of $CD4^+$ cells and $CD8^+$ cells in flow cytometry of cells immunized with peptide D^b 126 and non-immunized cells in Example 1.

[Figure 2]

Figure 2 is a graph comparing the cytotoxic effect on target cells immunized with peptide D^b 126 when pulsed with peptide D^b 126 and when not pulsed in Example 2.

[Figure 3]

Figure 3 is a graph with the same significance as Figure 2.

[Figure 4]

In Figure 4, A is a graph showing the cytotoxic effect of CTL induced by using peptide D^b 126 inT2 cells pulsed with peptide D^b 126 peptide in Example 3, and B is a graph showing the results of the cytotoxic effect of CTL induced by peptide WH 187 in T2 cells pulsed with peptide WH 187 in Example 3.

[Figure 5]

Figure 5 is a chart showing the results of FACS analysis of CTL surface markers induced by peptide D^b126 (CD19 cells and CD3 cells).

[Figure 6]

Figure 6 is a chart similar to Figure 5 for CD4 cells and CD8 cells.

[Figure 7]

Figure 7 is a chart similar to Figure 5 for CD56 cells.

[Figure 8]

Figure 8 is a chart showing the results of FACS analysis of CTL surface markers induced by peptide WH 187 (CD19 cells and CD3 cells).

[Figure 9]

Figure 9 is a chart similar to Figure 8 for CD4 cells and CD8 cells.

[Figure 10]

Figure 10 is a chart similar to Figure 8 for CD56 cells.

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Fig. 1

Immunized with D^b 126 peptide (Unstained) Non-immunized

Fig. 2

Mouse 1

[y-axis] Cytolysis (%)

[x-axis] E/T

Peptide D^b 126

No peptide

Mouse 2

[y-axis] Cytolysis (%)

[x-axis] E/T

Peptide D^b 126

No peptide

Fig. 3

Mouse 3

[y-axis] Cytolysis (%)

[x-axis] E/T

Peptide D^b 126

No peptide

Mouse 4

[y-axis] Cytolysis (%)

[x-axis] E/T

Peptide D^b 126

No peptide

Fig. 4

[y-axis] Cytolysis (%)

Peptide

[y-axis] Cytolysis (%)

Peptide

Fig. 5

Non-stained

Fig. 6

Non-stained

Fig. 7

Fig. 8

Non-stained

Fig. 9

Non-stained

Non-stained

Fig. 10

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[Document] Abstract

[Abstract]

[Problem] To offer novel cancer antigens

[Means for solving the problem] Cancer antigens comprising a product of Wilms' tumour suppressor gene *WT1* or 7-15 consecutive amino acids within said amino acid sequence including an anchor amino acid for binding with major histocompatibility complex (MHC) class I.

[Selected drawing] None

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[Document] Corrected data on power

[Document Corrected] Patent application

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